Molecular cloning and characterization of RBCK2, a splicing variant of a RBCC family protein, RBCK1

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Abstract RBCK1 (RBCC protein interacting with PKC 1) has two coiled-coil regions, a RING finger, a B-box and a B-box-like motif. RBCK2, a cDNA fragment related to RBCK1 was obtained, that lacks the 161-bp sequence of RBCK1 and encodes 260 amino acid residues. The 240-amino acid sequence in the NH_2-terminal of RBCK2 is identical with RBCK1 and contains two coiled-coil regions but no other structural motifs, whereas the 20-amino acid sequence in the COOH-terminal is distinct from RBCK1. The analysis of genomic DNA revealed that RBCK1 and RBCK2 are generated from a single gene by alternative splicing. The RBCK1 protein interacted with the RBCK1 and RBCK2 proteins, but the RBCK2 protein did not interact with itself, in vitro. The RBCK2 protein fused with the DNA-binding domain of yeast GAL4 (GAL4DBD) did not show a transcriptional activity, but the RBCK2 protein inhibited the transcriptional activity of the RBCK1 protein fused with GAL4DBD. These results suggest that RBCK2 may inhibit the transcriptional activity of RBCK1 probably through complex formation with RBCK1.

Key words: RBCC protein; Alternative splicing; Coiled-coil; Transcription factor

1. Introduction

The RBCC (RING, B-Box, Coiled-coil) protein family shares a common molecular organization, NH_2-(RING finger)-(B-box)-(Coiled-coil)-COOH [1], and about 10 proteins including efp [2], PML [3], T18 [4], rfp [5] and XNF-7 [6], have been identified as the family proteins. The precise physiological role of these RBCC proteins has not been elucidated, however, it is presumed that RING-zinc finger [7], B-box zinc-binding domain [8] and coiled-coil region [9] mediate protein-protein interactions. By the yeast two-hybrid screening using the regulatory domain of protein kinase C βI as a bait, a novel protein kinase C-interacting protein was identified from a rat brain cDNA library [10]. Interestingly, the protein contains structural motifs such as two coiled-coil regions, a RING finger, a B-box and a B-box-like motif in the order from NH_2- to COOH-terminals. As the protein has a unique molecular organization similar to the RBCC protein family, the protein was designated RBCK1 (RBCC protein interacting with PKC 1). By fusion with the DNA-binding domain of a yeast transcription factor GAL4 (GAL4DBD), the RBCK1 protein was found to possess a transcriptional activity in mammalian cells. Recently, XAP3 (a human hepatitis B virus X protein-associated protein) was cloned, that enhances the transactivation of gene expression by the hepatitis B virus X protein [11]. The XAP3 protein shows more than 90% similarity to the RBCK1 protein; however, the NH_2-terminal end sequence of XAP3 is distinct from RBCK1. Comparison of the nucleotide sequences indicated that RBCK1 has an additional region which is absent in XAP3, and that the 5′-noncoding region of RBCK1 encodes a peptide sequence highly similar to the NH_2-terminal end region of XAP3. Therefore, it is suggested that RBCK1 and XAP3 are the splicing variants produced from a homologous gene of different mammalian species, that may have a role in transcriptional control. While the RBCK1 cDNA fragment was amplified by PCR from a rat cDNA library, a fragment smaller than RBCK1 was isolated. The cDNA fragment encodes a splicing variant of RBCK1, which is distinct from XAP3. In this study, we report molecular cloning and characterization of RBCK2, a novel splicing variant of RBCK1.

2. Materials and methods

2.1. DNA cloning

Two cDNA fragments of approximately 1.6 and 1.8 kbp were amplified from a rat brain cDNA library for yeast two-hybrid screening (pGAD10-based; Clontech) by two steps of PCR using the primers corresponding to the RBCK1 DNA sequence. The primers for the primary PCR are 5′-aagaccaagaaagcagaggag-3′ (sense, nucleotides 305–325 of RBCK1) and 5′-aacagatgcggctgtgggtcc-3′ (antisense, nucleotides 2160–2180 of RBCK1). The primers for the nested PCR are 5′-atggccctgagccttgcccgg-3′ (sense, nucleotides 326–346 of RBCK1) and 5′-ctcagggagcaagctgagggg-3′ (antisense, nucleotides 2140–2160 of RBCK1). Genomic DNA fragments were cloned by two steps of PCR from an adapter-ligated rat genomic DNA (Rat GenomeWalker Kit; Clontech). The primers used are as follows: AP1 primer (adapter-derived upstream, Clontech); AP2 primer (adapter-derived downstream, Clontech); β15-IN1, 5′-atccctgcctcgtaccagcctgatg-3′ (sense, nucleotides 965–989 of RBCK1); β15-IN2, 5′-taccagcctgatgaggaggagcgag-3′ (sense, nucleotides 977–1001 of RBCK1); β15-IC1, 5′-ccagggcatgagtaggtgttgtcaatg-3′ (antisense, nucleotides 1264–1290 of RBCK1); β15-IC2, 5′-tgtcaatgaaggggcaggatacctc-3′ (antisense, nu-

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Abbreviations: GAL4DBD, yeast GAL4-DNA binding domain; PCR, polymerase chain reaction; GST, glutathione *S*-transferase; CREB, cAMP response element-binding protein; PMSF, phenylmethylsulfonyl fluoride; RT-PCR, reverse transcription polymerase chain reaction; knt, kilonucleotides

The nucleotide sequence of RBCK2 and the partial genomic sequence of RBCK1 and RBCK2 have been submitted to DDBJ/EMBL/GenBank and assigned accession numbers AB011369 and AB007133, respectively.

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cleotides 1247–1271 of RBCK1). The first PCR was performed with AP1 primer and β15-IN1, or AP1 primer and β15-IC1 primer, and the second PCR was performed using AP2 primer and β15-IN2, or AP2 primer and β15-IC2 primer. The amplified DNA fragments were ligated to pCR2.1 vector (Invitrogen), and sequenced on both strands by using a DNA sequencer model 373S (Applied Biosystems). Prediction of coiled-coil regions [9] was carried out at the Swiss Institute for Experimental Cancer Research WWW site (http://ulrec3.unil.ch/software/COIL-form.html).

2.2. Detection of mRNA

Northern blot analysis was carried out by hybridizing the RNA blot containing approximately 2 μg of mRNAs from adult rat tissues (Clontech) with the ^{32}P-labeled DNA fragment of either a common sequence of RBCK1 and RBCK2 (nucleotides 326–486 of RBCK1) or a RBCK1-specific sequence (nucleotides 1042–1202 of RBCK1) under the conditions described [12]. The radioactivity in hybridized bands was analyzed by a Bio-Imaging Analyzer BAS2000 (Fuji). RT-PCR was carried out employing the first-strand cDNAs synthesized from poly(A)$^+$ RNAs purified from normal rat tissues by using QuickPrep mRNA Purification Kit and Ready-To-Go T-primed First-Strand Kit (Pharmacia). The primers for the primary PCR are 5′-tatggctttccaccagcctg-3′ (sense, nucleotides 569–589 of RBCK1) and 5′-tcgattctctgcgatggacac-3′ (antisense, nucleotides 1361–1381 of RBCK1). The primers for the nested PCR are 5′-ggaggcgctgcgccagtatga-3′ (sense, nucleotides 1021–1041 of RBCK1) and 5′-caggaaacgctggtagtcctc-3′ (antisense, nucleotides 1311–1331 of RBCK1). PCR was carried out for 20 cycles of denaturing at 94°C for 30 s, annealing at 55°C 1 min and elongation at 68°C for 90 s. A series of the mixture of RBCK1 and RBCK2 (RBCK1 cDNA/RBCK2 cDNA = 10 ng/10 pg, 1 ng/10 pg, 100 pg/10 pg, 10 pg/10 pg, 10 pg/100 pg) was used as control.

2.3. Expression plasmids

The full-length sequences of RBCK1 and RBCK2 were inserted into a pTB701-FLAG vector [12] to construct the expression vectors containing a FLAG-epitope tag at the NH_2-terminal end, and designated RBCK1-FLAG and RBCK2-FLAG, respectively. The fusion protein constructs of RBCK1 and RBCK2 containing GST at the NH_2-terminal end were made by using pGEX-4T-1 vector (Pharmacia), and designated GST-RBCK1 and GST-RBCK2, respectively. The full-length sequences of RBCK1 and RBCK2 were inserted into pM vector (Clontech) to construct the expression vectors of RBCK1 and RBCK2 proteins fused with GAL4DBD at the NH_2-terminal end, and designated RBCK1-GAL4DBD and RBCK2-GAL4DBD, respectively. pFA-CREB plasmid (Stratagene) was used for expression of CREB protein fused with GAL4DBD at the NH_2-terminal end (CREB-GAL4DBD).

2.4. In vitro binding assay

The lysate of *E. coli* DH5α expressing either GST-RBCK1 or GST-RBCK2 was applied onto a glutathione-Sepharose 4B column (Pharmacia), and the GST-fusion protein was eluted according to the manufacturer's protocol [10]. The eluate was subjected onto Mono Q column (HR 5/5, Pharmacia), and the proteins were eluted by a linear concentration gradient of NaCl (0–0.5 M) in the buffer (20 mM Tris-HCl at pH 7.5, 1 mM EDTA, 1 mM EGTA, 10 mM 2-mercaptoethanol, 0.3 mM PMSF). COS-7 cells (about 5×10^7 cells) expressing either RBCK1-FLAG or RBCK2-FLAG were suspended in 500 μl of lysis buffer (20 mM Tris-HCl at pH 7.5, 300 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM 2-mercaptoethanol, 10 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 1 tablet/50 ml complete protease inhibitor cocktail tablet (Boehringer Mannheim), 1% (v/v) Triton X-100). Cleared lysate was incubated with purified GST-RBCK1 or GST-RBCK2 (approximately 10 μg) immobilized onto 20 μl (50% slurry) of glutathione-Sepharose 4B beads. After incubation at 4°C for 1 h, the beads were washed four times with the lysis buffer containing 0.1% (v/v) Triton X-100, and subjected to 12.5% SDS-polyacrylamide gel electrophoresis. Western blot analysis was carried out using an anti-

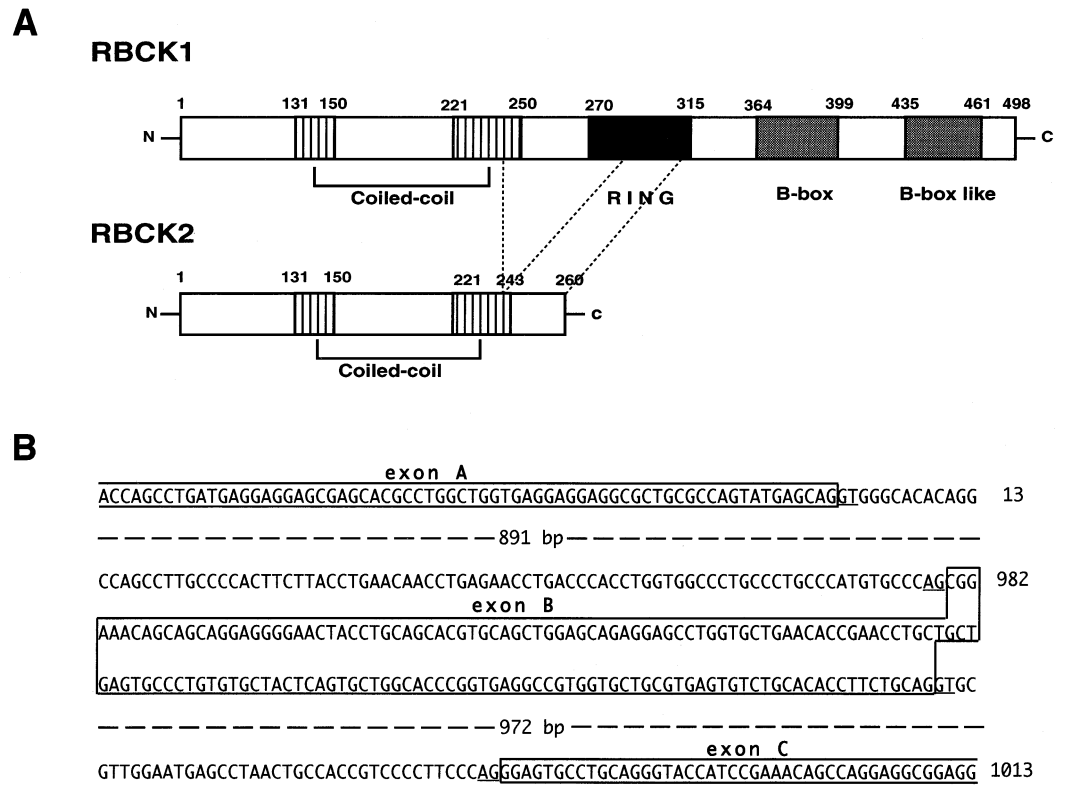

Fig. 1. Structure and genomic sequence of RBCK1 and RBCK2. A: Schematic drawing of RBCK1 and RBCK2. The amino acid residue numbers of the beginning and end of the proteins and the motifs are indicated on the top. The dotted lines indicate the correspondence of the nucleotide sequences. B: Partial genomic sequence encoding the divergent regions of RBCK1 and RBCK2. The sequence corresponding to the cDNA sequence of RBCK1 is boxed and indicated as exons A, B and C. The intron sequences between exons A and exon B (982 bp) and exons B and C (1013 bp) are abbreviated. Consensus sequences for 5′- and 3′-splice sites are underlined. Nucleotides of the intervening sequences are numbered on the right.

FLAG monoclonal antibody (M2, Eastman Kodak) as a primary antibody, and an alkaline phosphatase-conjugated anti-mouse IgG(H+L) antibody (Promega) as a secondary antibody [13].

2.5. Transcriptional activity

COS-7 cells (about 2.5×10^5 cells) were transfected with each transcription factor plasmid (50 ng; pM (GAL4DBD), RBCK1-GAL4DBD, RBCK2-GAL4DBD or CREB-GAL4DBD) and the reporter plasmid pFR-LUC (1 μg, Stratagene) which contains the luciferase gene from the firefly *Photinus pyralis* downstream of the synthetic promoter containing five copies of the 17-mer GAL4-recognition site. Where indicated, the RBCK2-expression plasmid (0–750 ng; RBCK2-FLAG) was co-transfected. After incubation for 24 h cells were washed once with phosphate-buffered saline and lysed with 200 μl of Passive Lysis Buffer (Promega). Each cell lysate (10 μl) was transferred into the tube containing 50 μl of the luciferase substrate solution (Promega). The luminescence originated from the firefly luciferase was measured as relative light units (RLU) by a luminometer (Lumat LB9507, EG&G Berthold). The transcriptional activity was defined as RLU/protein content of the sample.

3. Results and discussion

When the RBCK1 cDNA fragment of about 1.8 kbp encoding the full length of the RBCK1 protein (Met-1 to His-498) [10] was amplified by PCR from a rat brain cDNA library, an additional cDNA fragment of about 1.6 kbp was amplified simultaneously. The smaller cDNA fragment lacked the 161-bp sequence corresponding to nucleotides 1046–1206 of RBCK1 (accession number U48248), while the rest of the nucleotide sequence of these two cDNA fragments was identical. The smaller cDNA fragment has an open reading frame encoding a polypeptide of 260 amino acid residues consisting of the 240 amino acid residues (Met-1 to Gln-240) sharing with the RBCK1 protein and a divergent sequence of 20 amino acids followed by a stop codon (accession number AB011369). The smaller cDNA fragment designated RBCK2 encodes a protein with a calculated molecular weight of 29 086, which has two coiled-coil regions (amino acid residues 131–150, 221–243) but no zinc fingers such as RING finger and B-box (Fig. 1A). We isolated independent cDNA clones encoding RBCK2 from the rat brain cDNA library. The genomic DNA fragments encoding RBCK1 were isolated, and the DNA sequence encoding amino acid residues Tyr-218–Val-309 of the RBCK1 protein (nucleotides 978–1250 of the RBCK1 cDNA) was determined that involves the RBCK1-specific sequence of 161 bp (Fig. 1B, exon B). It was shown that the cDNA sequence of RBCK1 surrounding the 161-bp sequence was divided into three regions in the genomic DNA, and both 5′- and 3′-terminal end sequences of the two intervening regions completely fit with the consensus sequences of donor/acceptor splicing sites [14]; introns begin with GT and end with AG. No open reading frame having more than 50 amino acid residues was found in these intervening regions. These results indicate that these two intervening regions are introns and the RBCK1-specific sequence of 161 bp is an independent exon, and that RBCK1 and RBCK2 are transcribed from a single gene by alternative ways of splicing.

Northern blot analysis of rat tissues was carried out by using a probe common for RBCK1 and RBCK2 and a probe specific to RBCK1. An RNA transcript of about 2.4 kilonu-

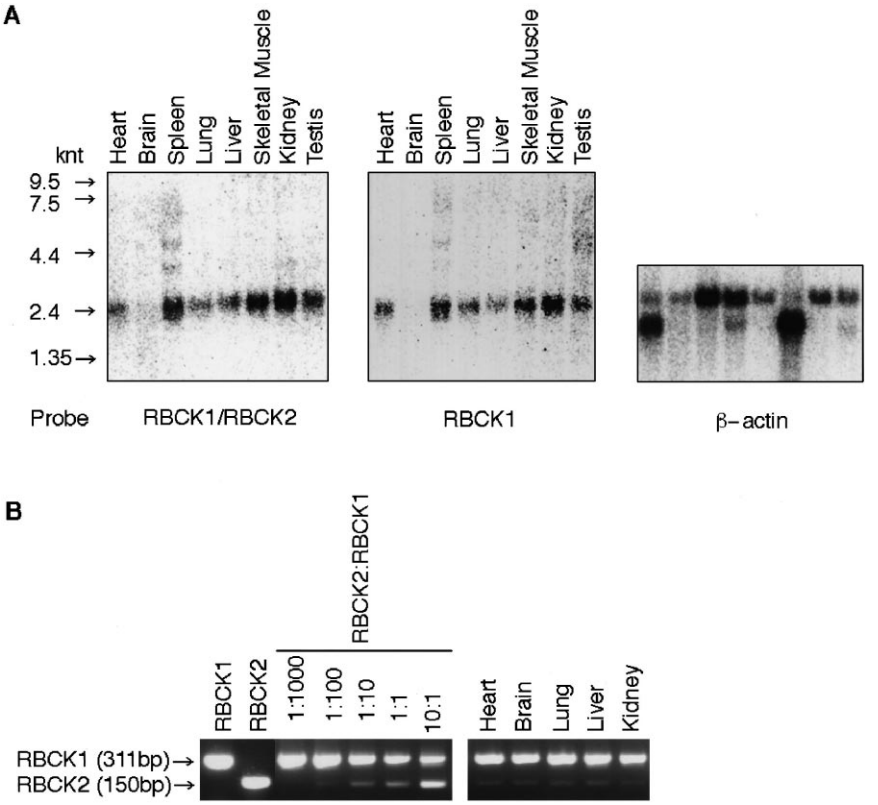

Fig. 2. Northern blot and RT-PCR of RBCK1 and RBCK2. A: Northern blot of RBCK1 and RBCK2 in normal rat tissues. Positions of size markers in kilonucleotides (knt) are shown on the left. The mutual sequence of RBCK1 and RBCK2 (left panel) and the insert sequence of RBCK1 (middle panel) are used as the common and RBCK1-specific probes, respectively. Rat β-actin is used as a control (right panel). B: RT-PCR analysis of RBCK1 and RBCK2 mRNA in rat tissues. cDNA of RBCK1, RBCK2 and a series of the mixture of RBCK1 and RBCK2 as indicated and RNA from rat tissues were used as template.

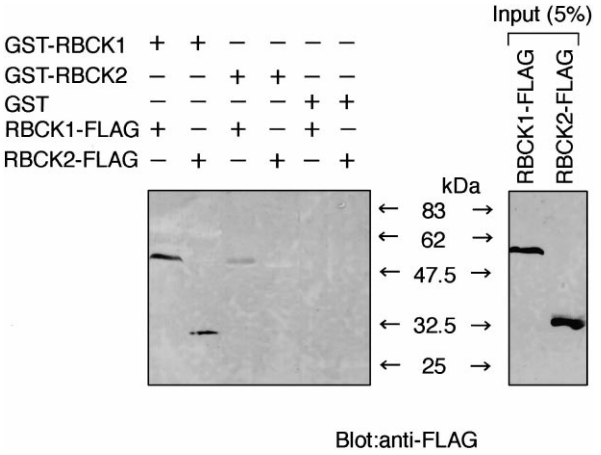

Fig. 3. Interaction between RBCK1 and RBCK2 proteins in vitro. The binding between RBCK1 and RBCK2 proteins was measured using GST-fusion proteins and FLAG-epitope tagged proteins. Bound proteins were eluted and analyzed by Western blot analysis using the anti-FLAG antibody. Positions of molecular weight markers are shown in the middle. Input lanes contain 5% of the proteins used in the binding assay.

cleotides (knt) was detected by both probes in all tissues examined (Fig. 2A). The expression of the 2.4-knt RNA transcript was low in brain, but the band was detected after longer exposure (data not shown). The expression of the RBCK1 and RBCK2 mRNA was investigated by RT-PCR (Fig. 2B). When PCR was carried out using a mixture of RBCK1 and RBCK2 cDNAs, RBCK2 was not amplified efficiently when RBCK1 existed, probably because RBCK1 prevents the amplification of RBCK2. The 150-bp fragment of RBCK2 was still observed in addition to the 311-bp fragment derived from RBCK1 in all rat tissues examined. In addition, both the RBCK1 cDNA and the RBCK2 cDNA were obtained as independent clones from a rat brain cDNA library (data not shown). The human XAP3 protein [11] is highly homologous to the RBCK1 protein. The 12-amino acid sequence of the NH_2-terminal end region of XAP3 protein is completely different from the corresponding region of the RBCK1 protein; however, the 5′-non-coding region of the RBCK1 cDNA (nucleotides 196–315) encodes a peptide highly similar to the NH_2-terminal region of the XAP3 protein. It seems that the RBCK1 protein and the XAP3 protein are splicing variants having distinct amino acid sequences in the NH_2-terminal end region. Therefore, at least three splicing variants, such as RBCK1, RBCK2 and XAP3 seem to be generated from a single gene. The 2.4-knt transcript in Fig. 2A may contain these RNA transcripts. It is worth noting that RBCK2 has another open reading frame starting from nucleotide 570, that encodes the 363-amino acid sequence having B-box and B-box-like motif but no coiled-coil region or RING finger. Further studies are required for the identification of the splicing variants derived from the RBCK1 gene.

The RBCK1 and RBCK2 proteins contain two coiled-coil regions, which are regarded to mediate the protein-protein interactions by forming amphipathic α-helix structures and are found in the dimerization elements of the transcription factors [9]. Thus, the interaction of RBCK1 and RBCK2 proteins was investigated. The lysate of COS-7 cells expressing either FLAG epitope-tagged RBCK1 or RBCK2 was mixed with the GST-RBCK1 or GST-RBCK2 protein, and Western blot analysis using the anti-FLAG antibody showed that GST-RBCK1 protein associated with both RBCK1-FLAG and RBCK2-FLAG proteins, and that the GST-RBCK2 protein associated with the RBCK1-FLAG protein, but not with the RBCK2-FLAG protein (Fig. 3). Association between the GST-RBCK2 protein and the RBCK2-FLAG protein was not observed, even when twice the amounts of the GST-RBCK2 protein were employed (data not shown). As the coiled-coil regions alone could not mediate homo-dimerization of the RBCK2 protein, the COOH-terminal half of the RBCK1 pro-

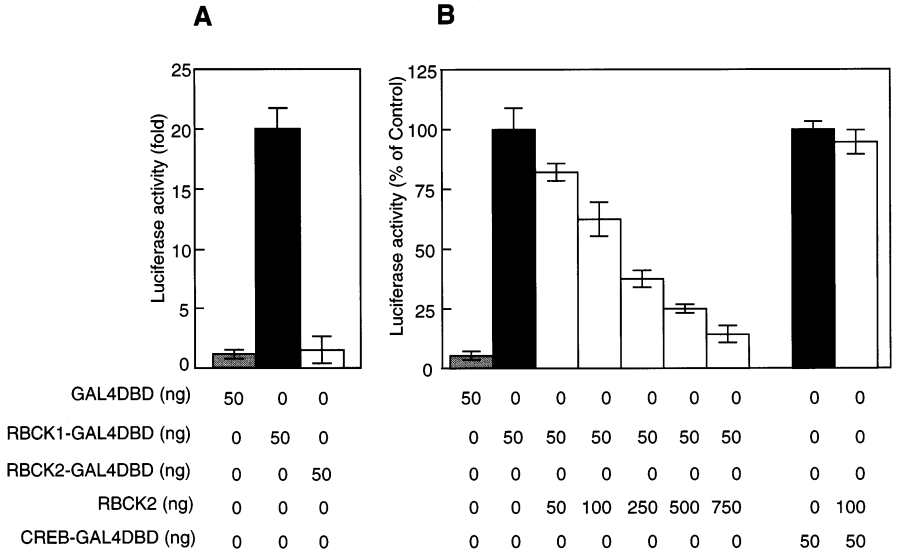

Fig. 4. Inhibition of transcriptional activity of RBCK1 by RBCK2. A: Transcriptional activity of RBCK1 and RBCK2. RBCK1-GAL4DBD and RBCK2-GAL4DBD were expressed and the transcriptional activities were measured. The data are shown as a ratio to the mean value of the activity in the control cells transfected with pFR-LUC and pM (GAL4 DBD). B: Effect of the expression of RBCK2 on the transcriptional activity of RBCK1 and CREB. RBCK1-GAL4DBD or CREB-GAL4DBD was expressed with RBCK2 and the transcriptional activity was measured. The transcriptional activity was calculated as a percentage of the mean value of the activity in the cells transfected with pFR-LUC and RBCK1-GAL4DBD or CREB-GAL4DBD. These results were of three independent experiments, each in duplicate, and the mean values with error bars ($P < 0.05$) are indicated.

tein including a RING finger, a B-box and a B-box-like motif may be important for the interaction of the RBCK1 protein and of the RBCK1 and RBCK2 proteins.

We have already reported that the RBCK1 protein has a transcriptional activity and the COOH-terminal half of the RBCK1 protein involves the transcription activating domain [10]. The transcriptional activity of the RBCK2 protein was examined by using a fusion protein of the RBCK2 protein and GAL4DBD (RBCK2-GAL4DBD) in COS-7 cells harboring the reporter plasmid containing the firefly luciferase gene downstream of the synthetic promoter containing the GAL4-recognition sequence. As shown in Fig. 4A, the RBCK2-GAL4DBD protein showed a luciferase activity of similar level as the control GAL4DBD protein, whereas the RBCK1-GAL4DBD protein showed about 20-fold higher luciferase activity than the GAL4DBD protein. When the RBCK2 expression plasmid was co-transfected with RBCK1-GAL4DBD, the transcriptional activity of the RBCK1-GAL4DBD protein was inhibited in a dose-dependent manner of the RBCK2 protein (Fig. 4B). On the other hand, the transcriptional activity of the GAL4DBD-fused CREB protein [15] was not affected by the overexpression of the RBCK2 protein. The RBCK2 protein may interact with the transcriptional activating domain of the RBCK1 protein, presumably in its COOH half. In several families of transcription factors, short coiled-coil regions of 2–5 heptads, such as a leucine zipper [16,17], mediate the homo- and hetero-dimerization of the factors. As the RBCK2 protein could not make a homo-dimer under the conditions employed, it seems possible that not only the coiled-coil regions but also other structural motifs such as the RING finger and the B-box may be involved in the interaction of the RBCK1 and RBCK2 proteins.

Some transcription factors are generated by alternative splicing mechanisms. In the case of the CREB family, eight species of CREB-related mRNAs are produced from a single gene by alternative splicings [18]. Only two species of them, CREBα and CREBΔ, show transcriptional activity, although the functions of other species have not been elucidated. It has been reported that an alternative AP-2 transcript (AP-2B) inhibits the activity of AP-2 transactivator [19]. Namely, AP-2B inhibits AP-2 transactivation through inhibition of its sequence-specific DNA binding, even though no direct interaction between AP-2A and AP-2B is observed. Recently, it has been reported that TRAF2 (tumor necrosis factor receptor-associated factor 2)-mediated NF-κB activation is inhibited by TRAF2A, a splice variant of TRAF2 that has a seven-amino acid insert within the RING finger domain of TRAF2 [20]. It is, however, still unknown whether TRAF2 and TRAF2A associate to inhibit NF-κB activation. In this study, the RBCK2 protein, a splicing variant of the RBCK1 protein, was revealed to inhibit the transcriptional activity of the RBCK1 protein, probably through the complex formation with the RBCK1 protein.

Recently, we have reported that both the RING finger and the B-box are indispensable for the transcriptional activity of RBCK1. However, the physiological role of these motifs of the RBCK1 protein is not yet clear [21]. The interaction between the RBCK1 and RBCK2 proteins might have some role in cellular function. To clarify the physiological roles of these proteins, it is important to elucidate the target genes and the associating proteins of the RBCK1 protein as a transcription factor.

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